

Chaperoned Ubiquitylation—Crystal Structures of the CHIP U Box E3 Ubiquitin Ligase and a CHIP-Ubc13-Uev1a Complex

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Summary

CHIP is a dimeric U box E3 ubiquitin ligase that binds Hsp90 and/or Hsp70 via its TPR-domain, facilitating ubiquitylation of chaperone bound client proteins. We have determined the crystal structure of CHIP bound to an Hsp90 C-terminal decapeptide. The structure explains how CHIP associates with either chaperone type and reveals an unusual asymmetric homodimer in which the protomers adopt radically different conformations. Additionally, we identified CHIP as a functional partner of Ubc13-Uev1a in formation of Lys63-linked polyubiquitin chains, extending CHIP's roles into ubiquitin regulation as well as targeted destruction. The structure of Ubc13-Uev1a bound to the CHIP U box domain defines the basis for selective cooperation of CHIP with specific ubiquitin-conjugating enzymes. Remarkably, the asymmetric arrangement of the TPR domains in the CHIP dimer occludes one Ubc binding site, so that CHIP operates with half-of-sites activity, providing an elegant means for coupling a dimeric chaperone to a single ubiquitylation system.

Introduction

Protein-ubiquitin ligases (E3s) facilitate transfer of ubiquitin from a covalent complex with a ubiquitin-conjugating enzyme (E2) to a target protein (or other ubiquitin molecule), with formation of an isopeptide bond between the C-terminal carboxyl of ubiquitin and a lysine side chain on the recipient. Target selectivity is provided by the E3, whereas specificity for the recipient ubiquitin lysine in formation of polyubiquitin chains may be determined by the E2 (Pickart and Eddins, 2004). The Lys48-linked chains that mark proteins for degradation via the proteasome (Hershko and Ciechanover, 1998) are generated by members of the Ubc4, Ubc5, and Ubc7 families for example, whereas formation of Lys63-linked chains, which act as a regulatory modification (Sun and Chan, 2004), requires a heterodimer of Ubc13 and either of the Ubc variants Uev1a or Mms2 (Hofmann

and Pickart, 1999). Mechanistically, E3 enzymes fall into one of two classes (Passmore and Barford, 2004). HECT domain E3 ligases have an active mechanism in which the ubiquitin C terminus is linked via a transient thioester bond to a reactive cysteine in the HECT E3 protein. RING-finger and the structurally related U box E3 ligases are passive and act as scaffolds, maintaining the target protein and E2-ubiquitin conjugate in proximity.

CHIP (C-terminal of Hsp70 interacting protein) is a dimeric ~35 kDa protein containing a C-terminal U box domain and an N-terminal tetratricopeptide repeat (TPR) domain that mediates its interaction with Hsp90 and Hsp70 chaperones. Originally characterized as a co-chaperone regulator of the ATPase cycle of Hsp70 (Balingier et al., 1999), in common with other U box proteins (Hatakeyama et al., 2001), CHIP has subsequently been shown to display E3 and E4 ubiquitin ligase activity (Jiang et al., 2001; Murata et al., 2001). The combination of chaperone binding and ubiquitin ligase activity suggests a role for CHIP in client protein quality control, facilitating the switch from chaperone-mediated folding/maturation to proteasome-mediated degradation via lysine 48-linked polyubiquitylation (Cyr et al., 2002; Wiederkehr et al., 2002). However, CHIP can also autoubiquitylate and facilitate polyubiquitylation linked via lysines other than lysine 48 (Alberti et al., 2002; Jiang et al., 2001; Murata et al., 2001).

To gain further insight into the mechanism of CHIP-mediated ubiquitylation and coupling to molecular chaperones, we have determined the crystal structure of CHIP in complex with a C-terminal decapeptide of Hsp90. Additionally, we have identified CHIP as a binding partner for the Lys63-specific ubiquitin-conjugating enzyme Ubc13-Uev1a, demonstrated that it collaborates as an E3 ubiquitin ligase, and determined the structure of a heterotrimeric complex between Ubc13-Uev1a and the CHIP U box domain. These studies explain CHIP's ability to interact with Hsp90 or Hsp70, define the basis for selective cooperation with specific ubiquitin-conjugating enzymes, and reveal an elegant means for coupling a dimeric chaperone to a single ubiquitylation system, by formation of an asymmetric homodimer.

Results

Structure of CHIP

Mouse CHIP was cocrystallized with a C-terminal peptide from human Hsp90 α , and the structure solved by molecular replacement using the structures of the CHIP U box domain dimer (see below) and the PP5 TPR-domain (Das et al., 1998) as search models (see Experimental Procedures and Table 1). Although the crystals only gave useful diffraction to 3.2 Å, virtually all residues are clearly defined, indicating that the limited diffraction is due to the thinness (<20 μ m) of the crystals obtained and their high solvent content (73% v/v), rather than significant disorder in the protein itself.

The N terminus of CHIP consists of a TPR-domain formed by three pairs of antiparallel α helices (residues 26–131), with an elongated seventh helix whose N

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Table 1. Crystallographic Statistics

	CHIP[166-304]- Ubc13-Uev1a	CHIP-Hsp90C10
Data Collection	ESRF ID14-eh3	ESRF ID14-eh3
Wavelength (Å)	0.931	0.980
Space group	C2	C2
Unit cell	a = 180.31 Å, b = 69.99 Å, c = 204.48 Å, β = 106.9°	a = 76.04 Å, b = 204.41 Å, c = 144.71 Å, β = 90.75°
Resolution (Å)	2.95	3.2
Observations (N)	146612 (21508)	109401 (14802)
Unique reflections (N)	49505 (7245)	31796 (4329)
Redundancy	3.0 (3.0)	3.4
Completeness (%)	96.1 (96.5)	98.0 (94.2)
R _{merge}	0.065 (0.312)	0.109 (0.512)
I/σI	12.2 (3.6)	4.2 (1.2)
Z	4	2
Solvent content (v/v %)	57	73
Refinement		
R _{cryst}	0.20	0.25
R _{free}	0.28	0.29

terminus packs against the third helical pair of the TPR repeats (Figure 1A). Helix 7 has a different structure in the two protomers of the CHIP dimer. In one, it is a straight α helix from Asp134 to Arg183. In the other, the polypeptide is broken into two separate and mutually perpendicular α helices, Asp134–Arg155 and Glu161–Arg183, connected by residues Ile156–Ser160 in an extended conformation. In both protomers, the chain reverses direction via a disordered loop (Asn184–Asp190) into another α helix (helix 8), which forms an antiparallel hairpin with the previous helix. As with the N-terminal side, the C-terminal side of the helical hairpin has a different conformation in the two protomers. In both cases, helix 8 is a continuous helix starting at Gly192 but ending at Lys224 in the protomer with the unbroken helix 7 and at Gln217 in the other. Beyond helix 8, in both cases, the polypeptide chain runs into a coil forming the beginning of the C-terminal U box domain, which has a similar structure in both protomers (Figure 1B). The U box domain consists of a pair of β hairpins (232–240 and 244–254) running into a short α helix (255–265), followed by a third hairpin (268–277) leading to a C-terminal α helix (274–298). Beyond the end of this helix, the two protomers again diverge; in the one with the continuous helix 7, the chain from 299 to the C terminus at 304 threads into a space between the N-terminal end of helix 7 (144–149) and the extended coil segment connecting the end of helix 8 to the beginning of the U box domain (226–289). In the other protomer, the N-terminal end of helix 7 is closely packed

against the beginning of the U box domain with no gap between them, so that the C terminus lies across the contiguous surface formed by these segments.

Architecture of the Asymmetric CHIP Homodimer

CHIP dimerization involves two separate segments of polypeptide: the U box domain and the distal segment of the helical hairpins (Figure 2A). The U box domain forms a parallel dimer, which buries >900 Å² of predominantly hydrophobic molecular surface, and the isolated U box domain shows some degree of dimerization in solution. The core of the interface is provided by a symmetrical interaction between the amide side chains of Asn284 from each protomer, buried between hydrophobic clusters (Tyr231, Leu285, Ala286, Met287, Val290, and Phe294 and Ile246, Pro248, Gly250, and Ile282), which pack with their equivalents in the other protomer. (Figures 2B and 2C). All residues involved are strongly conserved (Figure 1C). Apart from the extreme C terminus, the two crystallographically independent U box domains in the dimer are related by ~2-fold symmetry and are structurally very similar, with an rmsd on superposition of <0.5 Å over 67 C α positions.

The core of the second dimer interface is provided by a hydrophobic patch on the surface of the helical hairpin, involving Leu162, Tyr165, Leu166, Leu169, and Ile170 from the N-terminal arm of the hairpin and Tyr208, Met212, Leu215, Phe216, and Val219 from the C-terminal arm, which pack with their equivalents in the other protomer, forming a four-helix bundle. Corresponding residues from the two hairpins make equivalent contacts, so that the interacting regions are related by ~2-fold symmetry. However, the local 2-fold axis relating the helical hairpins does not coincide with the local 2-fold axis relating the U box domains but is tilted ~30° to it. The residues forming the second dimer interface are also strongly conserved in CHIP sequences, although the length of the hairpin varies between species. Consistent with the crystal structure, residues 128–229 from human CHIP in isolation were found to be substantially α -helical and dimeric in solution and their deletion substantially disrupts overall CHIP dimerization and E3 ligase activity (Nikolay et al., 2004).

An important consequence of the dislocation of the local dimer 2-fold axes, the breaking of the long helix in one protomer, and the different locations of the two C termini is that the two TPR domains have radically different positions with respect to their associated U box domain. Thus, if the U boxes are considered to be orientated East-West, the TPR domains are East-North (Figure 3A). In the elongated protomer, the TPR domain at the N-terminal end of the hairpin does not contact the U box at the C-terminal end, as the helices forming the

Figure 1. Structure of CHIP Protomers

(A) Secondary structure cartoon of the elongated CHIP protomer, rainbow colored (blue → red) from the N to C terminus, showing the tetratricopeptide-repeat domain (TPR) and the U box domain, linked via the long helical hairpin (HH). This and all other molecular graphics were produced by using MacPyMOL (<http://www.pymol.org>).

(B) As in (A) but for the compact protomer, with the U box domain in the same orientation. Rather than a continuous helix as in (A), the N-terminal arm of the helical hairpin (HH') is broken into two perpendicular segments.

(C) Alignment of exemplary CHIP sequences from mouse (Mm), human (Hs), chick (Gg), fish (Dr), fly (Dm), worm (Ce), and plant (At), with the secondary structures of the two chains in the mouse CHIP homodimer. Identical residues are in red, conserved residues are in yellow. The segments comprising TPR, helical-TPR, and U box domains are boxed in blue, green, and red, respectively. This figure was produced by using ESPript 2.2 (<http://esprict.ibcp.fr>).



hairpin are splayed apart by the insertion of the C terminus between them. In the other protomer, the TPR domain packs directly against the U box domain, with the C terminus of helix 6 involved in polar interactions with the C terminus of the first U box α helix (256–265), whereas the N terminus of the broken helix 7 makes extensive polar and hydrophobic interactions along its full length with the N-terminal β -hairpin turn of the U box (232–240) and the preceding coil segment (224–232) (Figure 3B).

Hsp90 C-Terminal Binding to the CHIP TPR Domain

CHIP can bind the C terminus of either Hsp70 or Hsp90 (Ballinger et al., 1999; Connell et al., 2001) via its single TPR domain, whereas Hop/Sti1, another TPR protein able to bind both chaperones, utilizes different TPR domains to provide selective and exclusive binding to one or other chaperone (Scheufler et al., 2000). To understand the promiscuity of CHIPs TPR-domain, we co-crystallized CHIP with a peptide (NH₃⁺-DDTSRMEEDV-COO[−]) corresponding to the C-terminal ten residues of Hsp90 α . CHIP bound this and a seven residue peptide with K_Ds 2.8 and 2.2 μ M, respectively, as determined by isothermal titration calorimetry (ITC) (see [Experimental Procedures](#)), but bound significantly less tightly (K_D \sim 90 μ M) to a five residue peptide containing only the MEEVD sequence. CHIP bound full-length human Hsp90 with K_D 4.9 μ M, suggesting that most of the affinity of CHIP for Hsp90 resides in the TPR-domain-peptide interaction.

Difference Fourier maps showed clear density for a bound peptide in the groove defined by the concave surface of the TPR-domains. Although the resolution is only 3.2 Å, the peptide is present in both TPR domains of the CHIP dimer, and in both copies of the dimer in the asymmetric unit, so that 4-fold geometric restraints could be applied, giving high confidence for the refined conformation. The main chain and side chain carboxyls of the C-terminal Asp731 of the Hsp90 α peptide make polar interactions with the side chains of Lys31, Asn35, Asn66, and Lys96 in the CHIP TPR domain, similar to the interactions made by Asp731 with Lys229, Asn233, Asn264, and Lys301 in the section TPR-domain of Hop (Scheufler et al., 2000). Similarly, the hydrophobic interaction of Hsp90 Val730 with the side chains of Phe38 and Leu69 in CHIP reflects its interaction with Tyr236 and Ala267 in Hop, whereas the side chain of Hsp90 α Glu729 has a polar interaction with Lys96 reminiscent of a weaker interaction with Lys301 in Hop. Consistent with the similar side chain interactions, this terminal EVD Hsp90 tripeptide segment has a similar main chain conformation in the two different TPR-domain complexes (Figure 4A).

Upstream of Glu729, the CHIP bound peptide departs substantially from the Hop bound conformation. Rather than an extended conformation, the main chain at Hsp90 residues Met727 and Glu728 makes a helical turn, bringing the side chain of Glu728 and the peptide nitrogen of Met727 into hydrogen bonding contacts with the side chains of CHIP Lys73 and Asp135, respectively. This backbone “kink” points the side chain of Met727 in the opposite direction to the Hop complex and buries it in a hydrophobic pocket formed by the side chains of Lys96, Phe99, Phe100, Phe132, and Ile136. This pocket

is a distinctive feature of the CHIP TPR and is not present in Hop. Upstream of Met727, the Hsp90 peptide chain is no longer bound within the channel of the TPR-domain but runs out across the loop connecting the end of helix 6 to the beginning of helix 7. The hydrogen bond to the peptide nitrogen of Met727 stabilizes the twisting of its side chain into the hydrophobic pocket, and its absence probably explains the weaker binding of the minimal MEEVD pentapeptide compared to longer sequences. Residues upstream of Met727 make no specific contacts with CHIP and are less well ordered.

The difference in bound peptide conformations explains how CHIP can bind either Hsp90 or Hsp70. The extended conformation in the Hop bound structures keeps chaperone side chains upstream of EEDV in contact with the TPR domains, which provide specific and exclusive interactions for one or the other sequence. In CHIP, the hydrophobic pocket provides a binding site that accommodates either the methionine of Hsp90 (DDTSRMEEDV) or the isoleucine of Hsp70 (GSGPTIEEDV) and, in doing so, twists the peptide into a conformation whereby upstream residues, which differ between the two chaperones, are hoisted clear of the TPR-domain groove so that no further specific contacts are required (Figure 4B).

Identification of CHIP as a Ubc13-Uev1a Interacting Protein

The E2 ubiquitin-conjugating enzyme Ubc13-Uev1a directs formation of Lys63 linkages (Hofmann and Pickart, 1999) and collaborates with TRAF RING-domain E3 ligases in formation of Lys63-linked polyubiquitin chains downstream of signaling through tumor necrosis factor, interleukin 1, and Toll-like receptors (Shi et al., 1999) (Deng et al., 2000; Wang et al., 2001). To identify proteins other than TRAFs that interact with Ubc13-Uev1a, we attached GST-Ubc13-Uev1a complex to glutathione Sepharose and incubated the beads with 293 cell extracts (see [Experimental Procedures](#)). After extensive washing, the Ubc13-Uev1a complex and associated proteins were released and separated by SDS-PAGE. A protein band of \sim 38 kDa (Figure 5A, top) was trypsinized and identified as human CHIP by mass spectrometry. This identification was strengthened by immunoblotting using an anti-CHIP antibody. To determine whether the association of CHIP with Ubc13-Uev1a was direct, we analyzed the interactions of the purified proteins *in vitro*. We confirmed that CHIP does indeed form a complex with Ubc13-Uev1a, that CHIP binds to Ubc13 and not to Uev1a, and that Ubc13 can bind Uev1a and CHIP simultaneously in a heterotrimeric complex (Figure 5B). To determine whether native Ubc13 and CHIP interact *in vivo*, we transfected 293 cells with GST-Uev1a and analyzed precipitated proteins as above. Immunoblotting clearly shows coprecipitation of Ubc13 and CHIP with GST-Uev1a, confirming the existence of an endogenous Ubc13-CHIP *in vivo* (Figure 5C).

CHIP-Ubc13-Uev1a Catalyzes Formation of Lys63-Linked Polyubiquitin Chains *In Vitro*

As CHIP was known to be a ubiquitin E3 ligase, its interaction with Ubc13-Uev1a suggested that a Uev1a-Ubc13-CHIP complex might catalyze formation of Lys63-linked polyubiquitin chains. This idea was

A



Figure 2. Architecture of the CHIP Homodimer

(A) Secondary structure of the CHIP homodimer, with each protomer rainbow colored as in [Figure 1](#). The Hsp90 C-terminal decapeptide bound to each TPR domain is shown in magenta. Distinct dimer contacts occur between the two U box domains and between the distal ends of the two helical hairpins, which form a four helix bundle.

(B) View of the CHIP U box dimer showing side chains of residues in one chain packed against the molecular surface of the other chain. The CHIP U box dimerization interface is primarily formed by mutual packing of residues projecting from the C-terminal helix of each monomer, with additional interactions from residues in the first and second hairpin loops.

(C) The amide head group of Asn284 forms a symmetrical hydrogen-bond interaction with its equivalent in the other monomer and lateral interactions with Thr252 in the same monomer. The strength of this polar interaction will be significantly enhanced by its burial in the hydrophobic core of the interface.

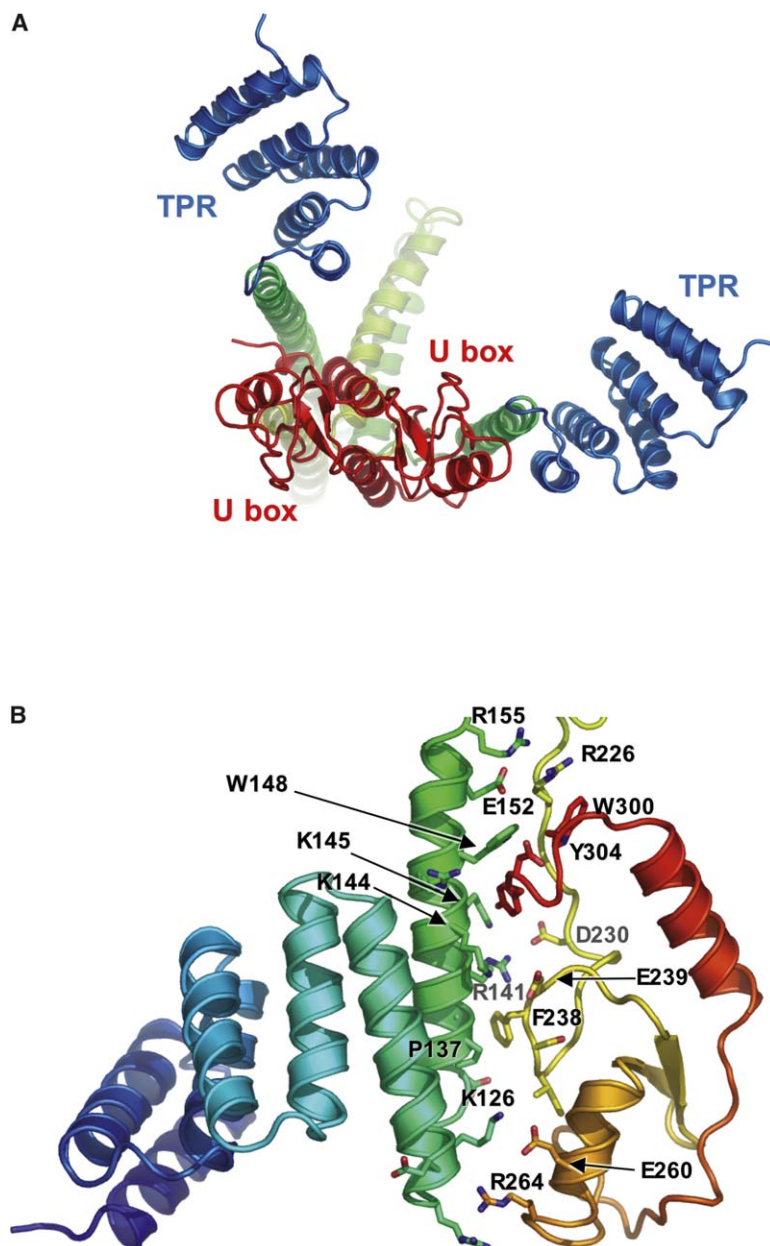


Figure 3. Asymmetric Disposition of TPR Domains

(A) The CHIP dimer viewed along the local 2-fold relating the two U box domains (red) clearly show the dramatically different relative location of the two TPR domains (blue). The TPR domain of the elongated protomer makes no direct contact with its associated U box, whereas in the other protomer, the TPR and U box domains are packed together. (B) Details of the interface between the TPR and U box domains of the compact protomer, consisting of polar interactions surrounding a core hydrophobic interaction of Phe238 packing between the side chains of Pro137, Arg141, and Lys144.

validated by carrying out ubiquitylation reactions in vitro in which CHIP, Ubc13-Uev1a, and UBE1 catalyzed formation of polyubiquitin chains (Figure 5D, top panels). Protein staining or immunoblotting failed to detect any ubiquitin-CHIP conjugates, indicating that the polyubiquitin chains are not attached covalently to CHIP. In contrast, CHIP paired with the E2 UbcH5a formed polyubiquitin chains anchored to CHIP, as indicated by a ladder of ubiquitin-CHIP conjugates (Figure 5D, bottom panels). To test whether the polyubiquitin chains formed by CHIP-Ubc13-Uev1a are linked via Lys63, we used ubiquitin mutants with either Lys48 or Lys63 mutated to Arg (Figure 5E). As expected, CHIP-Ubc13-Uev1a formed polyubiquitin chains with either wild-type ubiquitin [wt] or ubiquitin[R48], but not with ubiquitin[R63]. In contrast, the CHIP-UbcH5a complex formed polyubiquitin chains with all three ubiquitin species,

suggesting that it is not specific for either Lys48 or Lys63 in vitro.

Structure of CHIP-Ubc13-Uev1a Complex

To understand the interaction of CHIP and Ubc13-Uev1a at a structural level, the purified proteins were cocrystallized. Full-length CHIP with Ubc13 or Ubc13-Uev1a yielded crystals; however, none gave useful diffraction. A series of N-terminal deletion mutants were constructed, with a construct consisting of residues 166–304 (C terminus) producing diffracting crystals when cocrystallized with Ubc13-Uev1a. The structure of the mCHIP[U]-hUbc13-hUev1a (CUU) complex was determined by molecular replacement using the structure of the human Ubc13-Mms2 complex (Moraes et al., 2001) and refined at 2.9 Å resolution (see Experimental Procedures).

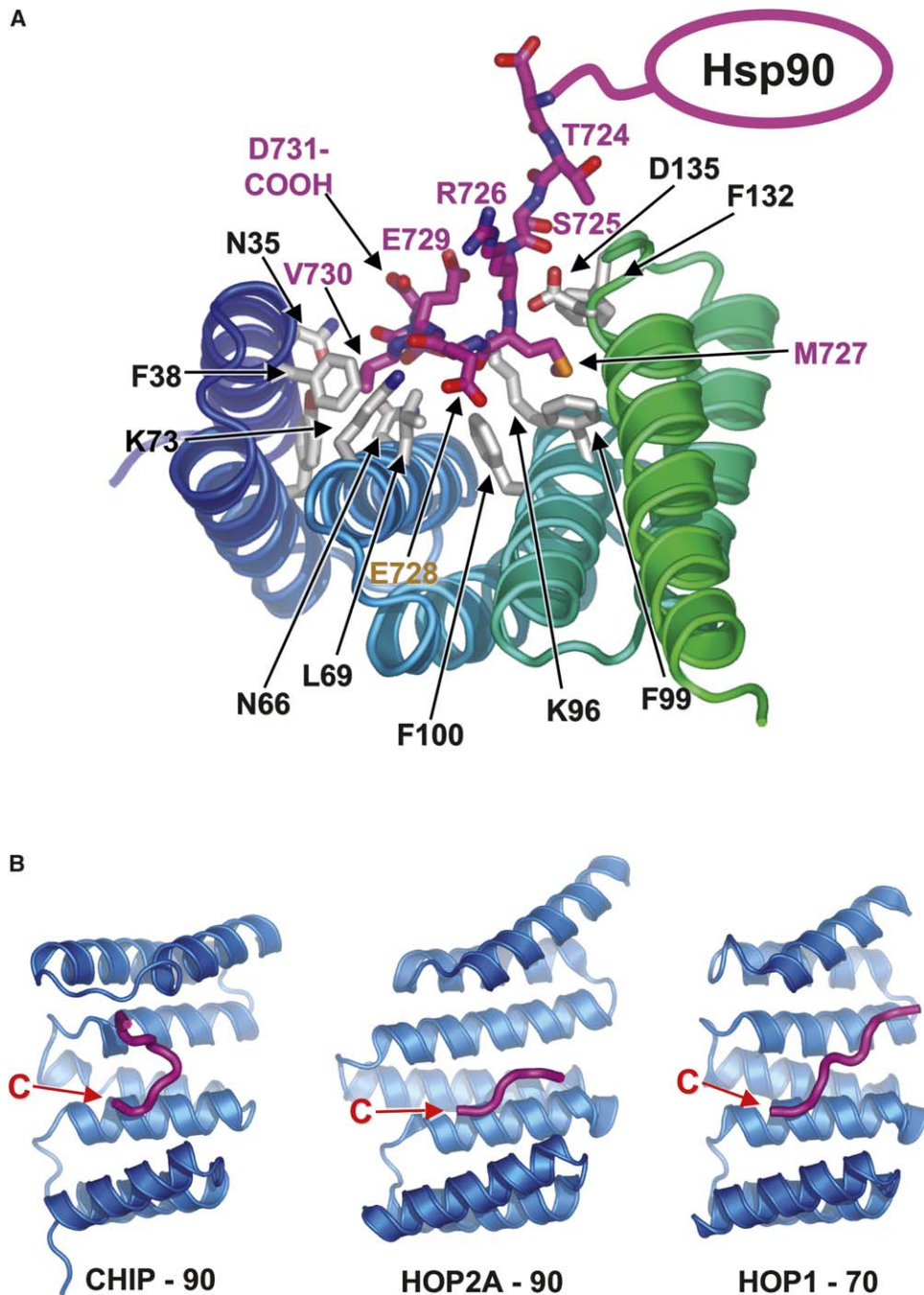


Figure 4. CHIP TPR Peptide Binding

(A) Detailed view of Hsp90 C-terminal decapeptide bound to the CHIP TPR domain. Whereas the C-terminal EVD sequence lies in an extended conformation along the TPR channel, the side-chain of Met727 binds in a hydrophobic pocket, twisting the upstream residues out of the TPR channel and free of specific interactions.

(B) Comparison of chaperone C-terminal peptide conformations (magenta) bound to cochaperone TPR domains. CHIP - Hsp90 C10 (left), Hop (TPR2A) - Hsp90 C5 (middle), and Hop (TPR 1) - Hsp70 C10 (right).

The CUU complex consists of a homodimer of the CHIP U box domain with a Ubc13-Uev1a heterodimer bound on either side (Figures 6A and 6B). Although the CHIP construct comprised residues 166–304, clear electron density is only visible for residues 225–304, which encompass the U box domain. Silver-stained PAGE of dissolved crystals (data not shown) suggests that residues 166–224 are present but disordered. The structure

of Ubc13 in the CUU complex is essentially identical to the structure in complex with Mms2 alone (Moraes et al., 2001; VanDemark et al., 2001). In particular, a putative catalytic residue Ubc13 Asn79 remains hydrogen bonded to the peptide backbone and is not moved by CHIP binding as has been suggested (Wu et al., 2003). Uev1a is similar in structure to Mms2 but with additional N-terminal residues that are mostly disordered in the

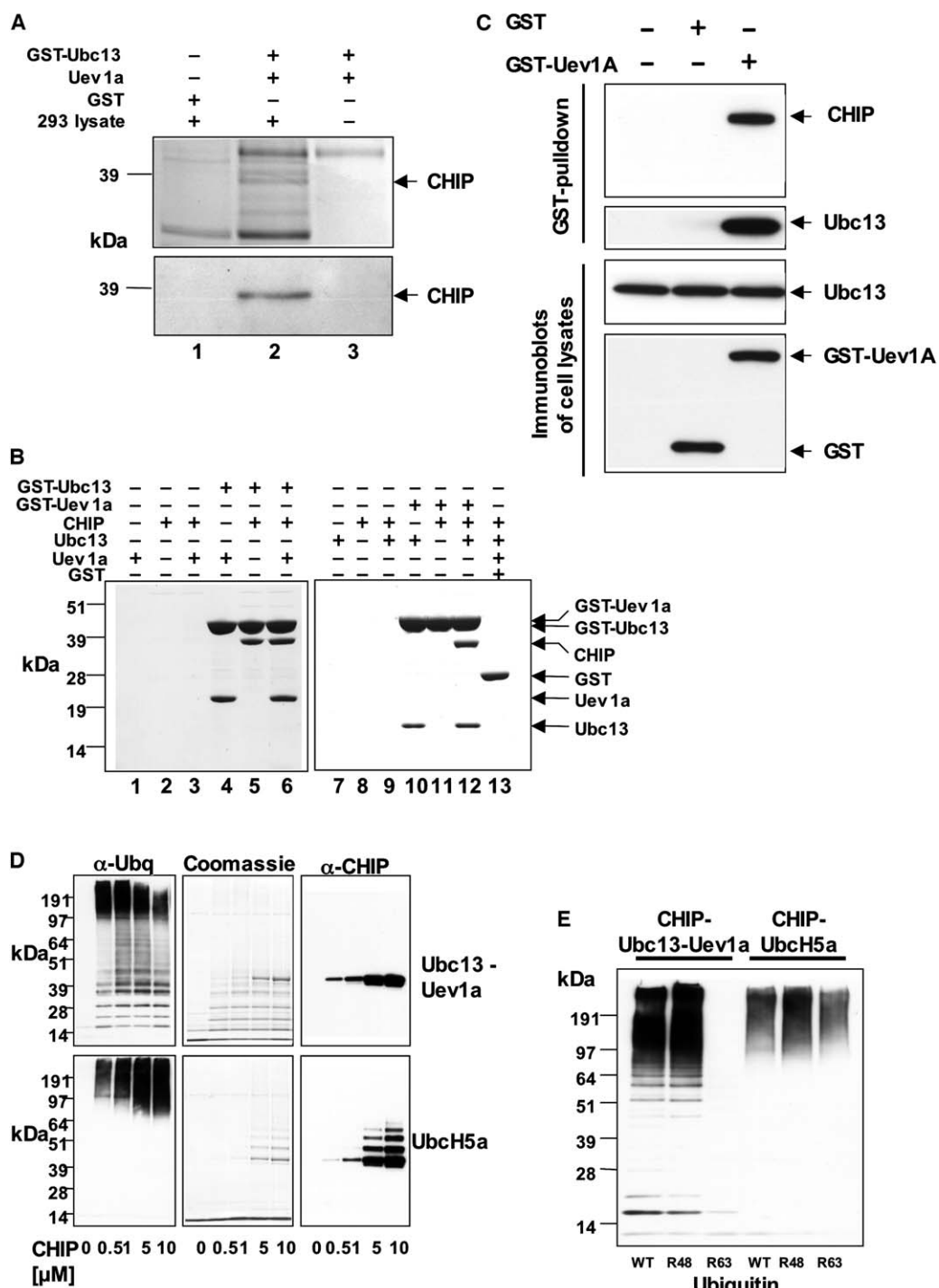


Figure 5. Binding and Ubiquitylation Activity of CHIP and Ubc13-Uev1a

(A) Identification of CHIP as a Ubc13-Uev1a binding protein in 293 cells. GST-Ubc13 and Uev1a or GST were immobilized on glutathione Sepharose beads and incubated with 293 cell extracts. The Ubc13-Uev1a complex was released from the beads by treatment with PreScission protease. After concentration, the eluted proteins were subjected to SDS PAGE and the gel either silver stained (top) or transferred to a nitrocellulose membrane and immunoblotted with antibodies that recognize CHIP (bottom). The silver-stained band was excised and identified as CHIP by mass spectrometry after digesting the protein with trypsin.

(B) Bacterially expressed CHIP binds to Ubc13, but not to Uev1a, in vitro. GST-Ubc13, GST-Uev1a, and GST-CHIP were expressed in bacteria and purified. Where indicated, GST tags were removed by treatment with PreScission protease (see Experimental Procedures). The purified proteins (each at 4 μM) were incubated for 1 hr at 4°C with 25 μl glutathione Sepharose beads in 0.1 ml of lysis buffer. The beads were washed three times with 1 ml of lysis buffer and once with 1 ml of 10 mM Tris-HCl (pH 8). The bound proteins were released by denaturation in SDS, subjected to SDS-PAGE, and stained with Coomassie blue.

(C) 293 cells transfected with pEBG6P (GST) or pEBG6P-Uev1A (GST-Uev1A) were extracted with lysis buffer and proteins precipitated by addition of glutathione Sepharose beads. After 1 hr at 4°C, the beads were washed four times with 1 ml lysis buffer and once with 1 ml 10 mM

CUU complex. The relative orientations of Ubc13 and Uev1a and their interface are similar to those observed between Ubc13 and Mms2.

Structural Determinants of CHIP Specificity for E2 Enzymes

Each CHIP U box domain in the dimer interacts independently with a Ubc13 molecule, which itself interacts with a Uev1a molecule (Figures 6A and 6B). The CHIP-Ubc13 E3-E2 interface in the CUU complex is similar in overall structure to that in the C-Cbl-UbcH7 E3-E2 complex (Zheng et al., 2000), with equivalent elements in the CHIP U box and c-Cbl RING domains interacting with equivalent sites on the E2 surfaces (Figure 6C). The core interface involves a hydrophobic ridge on Ubc13 (Met64, Pro97, and Ala98) inserting into a hydrophobic groove on the U box surface, between the short α helix (254–265) and the tips of two hairpin turns (234–239 and 269–274). A second hydrophobic interaction is provided by the side chain of Phe238 from CHIP packing between the side chains of Arg7 and Lys10 from the N-terminal α helix of Ubc13 and the head group of Arg7. These interactions are supported by a network of polar contacts, including hydrogen bonds from the main-chain carbonyl oxygens of CHIP Ile236 and Pro270, with the side chains of Ubc13 Arg7 and Ser96 (Figure 6D). Analysis of Ubc sequences suggests that these interactions, along with the side chain of Ala98, determine the spectrum of Ubc enzymes with which CHIP can cooperate. In addition to the functional interaction with Ubc13-Uev1a shown here, CHIP also cooperates with Ubc4 and the UbcH5 E2 enzyme family, but not with E2-25K (HIP-2), UbcH2, UbcH3, or UbcH7 (Hatakeyama et al., 2001; Jiang et al., 2001; Murata et al., 2001). Ubc4 and UbcH5 enzymes have the Ser-Pro-Ala motif equivalent to residues 96–98 of Ubc13 and present a very similar E3 binding surface as Ubc13. UbcH7, by contrast, replaces the serine with lysine, which could not be accommodated in the close-packed interface with CHIP. Similar incompatibility is presented by UbcH2 and UbcH3, which replace the Ser-Pro-Ala motif with Asn-Pro-Thr and Thr-Ala-Leu, respectively, whereas the Ala-Ala-Ala of E2-25k could not make the hydrogen bond with the carbonyl of CHIP Pro270. It has been reported that UbcH6 or UbcH8 cannot cooperate with CHIP, even though the Ser-Pro-Ala motif is conserved in these enzymes (Jiang et al., 2001; Murata et al., 2001). However, in our hands, CHIP polyubiquitin conjugates were formed readily in the presence of UbcH6 (M.W., unpublished data), consistent with the role proposed for these residues.

CHIP Asymmetry and Binding of Ubiquitin-Conjugating Enzymes

It is immediately clear when the CHIP U box dimer from the CUU complex is superimposed on the full CHIP structure (Figures 2A and 6A) that the TPR domain interacting with its attached U box domain overlaps the position of Ubc13 and would prevent binding of Ubc13-Uev1a to that U box domain. Indeed several of the U box residues involved in binding Ubc13 are buried in the interface between the U box and its associated TPR domain in the full structure. The other TPR domain makes no interaction with its associated U box and presents no obstacle to binding of Ubc13-Uev1a (Figure 7A). A necessary consequence of this structural observation is that the full CHIP dimer should only bind a single Ubc13 molecule. To test this, we measured the binding of Ubc13 to CHIP by ITC. In either orientation of the experiment (i.e., CHIP added to Ubc13 or Ubc13 added to CHIP), we consistently observed formation of a CHIP-Ubc13 complex with a 2:1 stoichiometry. However, with the truncated CHIP construct (166–304) that lacks a TPR domain but retains the ability to dimerize, we observed clear 1:1 binding, showing that both U boxes are competent in E2 binding in the absence of the TPR domains (Figures 7B, 7C, and 7D).

Discussion

The structure of CHIP provides a rare example of a homodimeric protein in which the protomers adopt significantly different conformations. In the few other described examples, homodimer asymmetry is concomitant with ligand binding, whereas in CHIP, it is an inherent property. An important consequence of this asymmetry, confirmed experimentally, is the blockade of one of the two binding sites for Ubc enzymes provided by the U box domains in the CHIP dimer. Thus, a CHIP dimer binds a single Ubc, displaying effective “half-of-sites” activity and resolving the conundrum of how two U box E3 ligase domains might cooperate in formation of a monotonic polyubiquitin chain. Although the two TPR domains occupy different locations in the dimer, they are both competent for Hsp90 C-terminal peptide binding, so that CHIP and Hsp90 form a dimer-dimer complex. The asymmetric dimer structure of CHIP provides an elegant means for coupling a single ubiquitin conjugation system to a dimeric chaperone (Figures 7E and 7F).

Since CHIP was recognized as an E3-ubiquitin ligase (Jiang et al., 2001), it has been implicated in polyubiquitylation and proteasomal targeting of a range of

Tris-HCl (pH 8). The bound proteins were released by denaturation in SDS, subjected to SDS-PAGE, and immunoblotted with antibodies against CHIP and Ubc13 (upper two panels). The expression of CHIP and Ubc13 was analyzed by immunoblotting after SDS-PAGE by using 20 μ g cell lysate protein (bottom two panels). Coprecipitation of endogenous Ubc13 and CHIP occurs when GST-Uev1a is expressed, but not with GST only. (D) Polyubiquitylation was carried out for 1 hr at 30°C in an assay comprising 20 μ l of 50 mM Tris-HCl (pH 7.5), His₆-E1 (0.1 μ M), 1 μ M Ubc13/Uev1a (top row) or 1 μ M UbcH5a (bottom row), ubiquitin (100 μ M), and CHIP was as indicated, 5 mM MgCl₂, 2 mM ATP. The reaction was initiated with ATP, stopped by addition of SDS, subjected to SDS PAGE, stained with Coomassie blue (center column), or transferred to nitrocellulose and immunoblotted by using anti-ubiquitin (left column) or anti-CHIP antibodies (right column). Maximal polyubiquitylation with CHIP-Ubc13-Uev1a occurs at 1 μ M CHIP, whereas polyubiquitylation with CHIP-UbcH5a continues to increase up to 10 μ M CHIP. (E) Polyubiquitin chains synthesized by CHIP-Ubc13-Uev1a are linked via Lys63, whereas those synthesized by CHIP-UbcH5a do not use Lys48 or Lys63 exclusively. The reaction was performed as in (A) by using 1 μ M of Ubc13-Uev1a or 5 μ M UbcH5a, with ubiquitin[K48R] or ubiquitin[K63R] used instead of wild-type ubiquitin as indicated. Reaction products were subjected to SDS PAGE and immunoblotted with anti-ubiquitin antibodies.

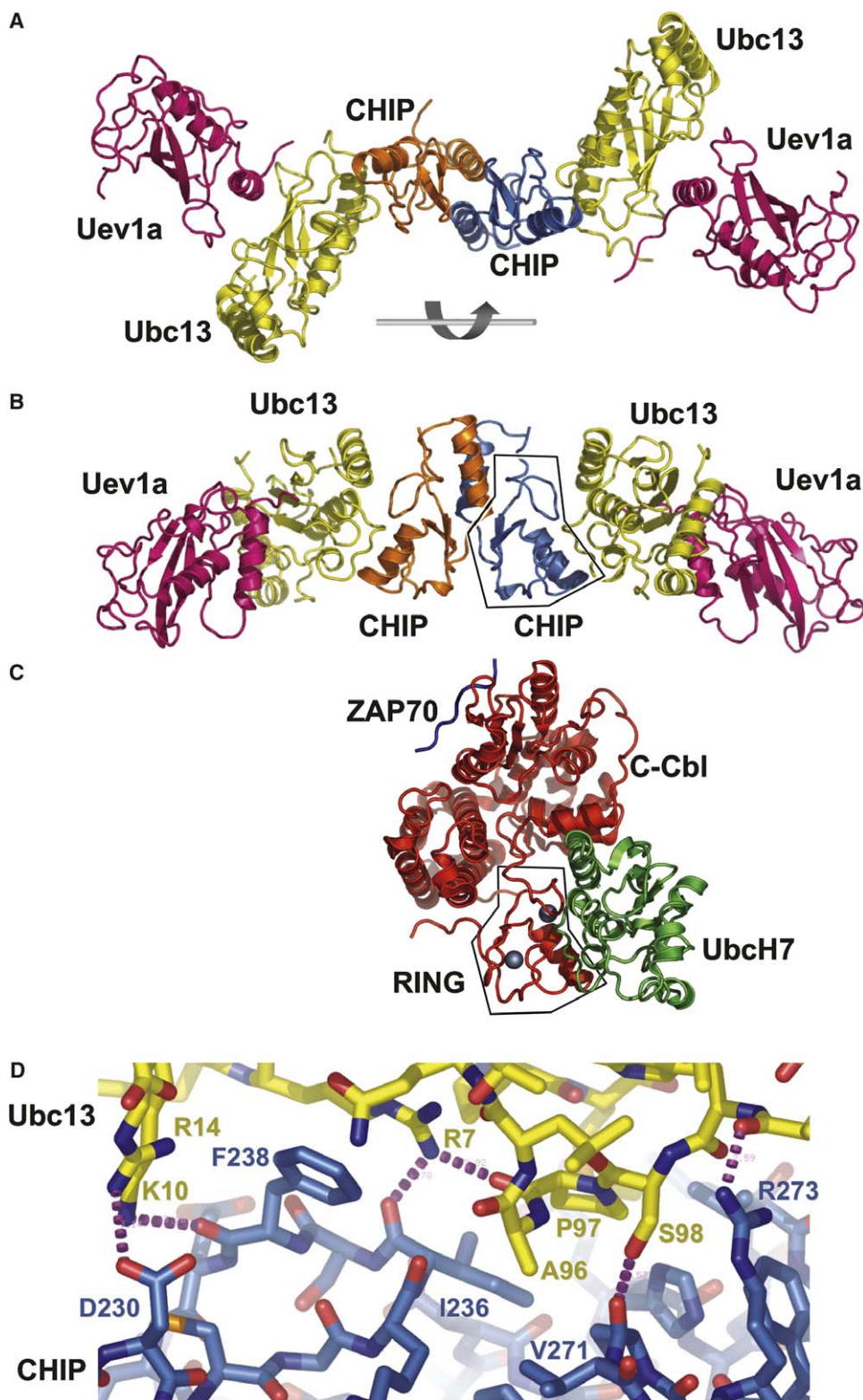


Figure 6. Structure of CHIP[U]-Ubc13-Uev1a Complex

(A) Secondary structure cartoon of the homodimerized CHIP[U]-Ubc13-Uev1a heterotrimeric complex viewed down the local 2-fold axis. Uev1a molecules are shown in pink, Ubc13 molecules in yellow, and the separate chains of the CHIP U box dimer in orange and blue.

(B) As in (A) but rotated 90° clockwise around the horizontal. One U box domain is outlined.

(C) The RING domain in the c-Cbl-UbcH7 complex (outlined) interacts with the same region of the E2 enzyme as the U box domain of CHIP, although the detailed interactions are different and E2 specific.

(D) Detail of the core interaction between CHIP (blue carbons) and Ubc13 (yellow carbons). Close interactions of residues 96, 97, and 98 in Ubc13 against the surface of CHIP define the spectrum of Ubc E2 enzymes that CHIP can collaborate with.

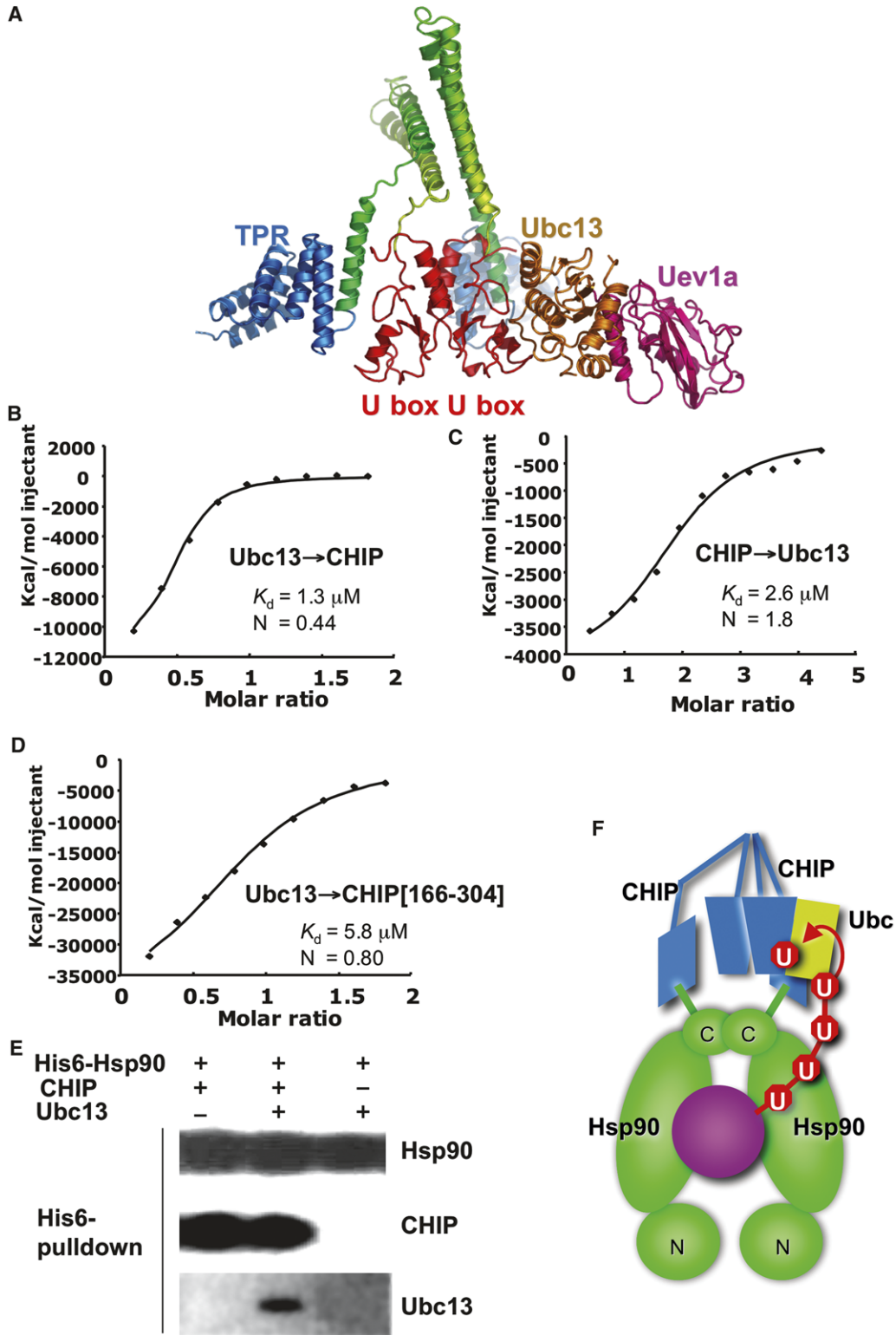


Figure 7. Half-of-Sites Activity in CHIP-Mediated Ubiquitylation

(A) Model of a full CHIP-Ubc13-Uev1a complex constructed by superimposing the U box dimer from the CUU complex on to the U box dimer in the full CHIP-peptide complex. The asymmetric disposition of the TPR domains blocks one of the Ubc binding sites on the U box domains; however, each TPR domain remains available to bind a C-terminal peptide from each protomer of an Hsp90 dimer.

(B) ITC curve showing heats evolved on adding aliquots of Ubc13 to CHIP. Least-squares fitting of the data indicate micromolar affinity for the interaction, with a stoichiometry ($N = 0.44$) consistent with formation of a Ubc13-CHIP₂ complex.

(C) As in (B) but for the inverse titration in which aliquots of CHIP were progressively added to Ubc13. Again, the stoichiometry ($N = 1.8$) is fully consistent with formation of a Ubc13-CHIP₂ complex.

proteins, including ErbB2 (Xu et al., 2002; Zhou et al., 2003), nitric oxide synthase (Jiang et al., 2003; Peng et al., 2004), microtubule-associated Tau protein (Hatakeyama et al., 2004; Petrucelli et al., 2004; Shimura et al., 2004), cystic-fibrosis transmembrane-conductance regulator (Meacham et al., 2001; Younger et al., 2004), SMAD proteins (Li et al., 2004), and E2A transcription factors (Huang et al., 2004), several of which are known Hsp90 clients. In collaboration with the E4 UFD2, *C. elegans* CHIP is also involved in ubiquitylation of an Hsp90 cochaperone, the myosin binding UNC45 (Hoppe et al., 2004). Here, we have shown that CHIP associates with Ubc13 in vivo and is able to promote formation of Lys63-linked polyubiquitin chains in collaboration with Uev1a-Ubc13 in vitro. This suggests that CHIP is not only a protein quality control factor, but it also provides the means for coupling regulatory Lys63 polyubiquitylation to the client proteins of molecular chaperones. To understand the significance of this in vivo, targets of CHIP-mediated Lys63 ubiquitylation need be identified. Lys63-linked polyubiquitylation is involved in a range of nondegradative processes, including receptor-mediated endocytosis (Galan and Haguenaue-Tsapis, 1997), error-free DNA repair (Hoege et al., 2002), ribosome stability (Spence et al., 2000), and signaling via the TNF/IL-1 Toll-like receptor system (Deng et al., 2000), which leads to the Lys63-linked polyubiquitylation of NEMO, a regulatory subunit of the IKK complex. CHIP involvement in this pathway is a particularly tantalizing possibility, as several components of this system have involvement with Hsp90 (Bouwmeester et al., 2004; Chen et al., 2002) and preliminary data (data not shown) suggest that CHIP is present in some IKK/NF- κ B-associated complexes. Nevertheless, considerably more work will be required to elaborate this possibility.

As CHIP can cooperate with different E2 enzymes templating different ubiquitylation topologies, with very different potential biological outcomes, it is likely that the choice of E2 will be regulated. Conceivably, the chaperone involved and the identity and/or state of the chaperone bound client protein, the presumed target of CHIP-directed ubiquitylation, might play a role in determining the type of E2 system recruited, but this remains to be shown. It is also unclear, even for the better characterized degradative ubiquitylation function of CHIP, what roles are played by Hsp90-CHIP and Hsp70-CHIP complexes, both of which exist in cells. With a denatured model protein, either type of chaperone-CHIP complex was able to promote target polyubiquitylation independently of the other (Murata et al., 2001). However, CHIP-dependent ubiquitylation of the bone fide Hsp90 client ErbB2 suggests a far more complex process in which enzymatically active CHIP is recruited to Hsp90-ErbB2 as a prerequisite for ubiquityla-

tion of the client and consequent transfer to an Hsp70-CHIP complex (Xu et al., 2002), again showing the close interdependence of the Hsp70 and Hsp90 chaperone systems.

Experimental Procedures

DNA Constructs

Human Ubc13, Uev1a, and CHIP were amplified from IMAGE ESTs (2822013, 6065716, and 3847168, respectively) and cloned into pGEX6P-1 (Amersham) for bacterial expression. FLAG CHIP was created in a similar manner with a 5' KOZAK sequence and 3' FLAG tag being added by PCR and cloned into pCMV5 for expression in mammalian cells. DNA expressing ubiquitin, ubiquitin[K48R], and ubiquitin[K63R] were cloned into pGEX6P-1. cDNA encoding UBE1 was amplified, cloned into pCR2.1, and subcloned into the BamHI site of EBG2T. Mouse CHIP and various subconstructs were amplified from pooled cDNA and cloned into pRSETA.

Protein Expression and Purification

His₆-tagged ubiquitin-activating enzyme 1 (UBE1) was expressed in Sf21 cells and purified on Ni-NTA agarose. Ubc13, Uev1a, hCHIP, ubiquitin, ubiquitin[K48R], and ubiquitin[K63R] were expressed in *E. coli* with N-terminal GST tags and purified on glutathione Sepharose (Pharmacia). GST tags were removed and proteins released by cleavage with PreScission Protease (Pharmacia). Proteins were dialyzed against 50 mM Tris-HCl (pH 7.5), 270 mM sucrose, 150 mM NaCl, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 0.2 mM PMSF, and 1 mM benzamidine and stored at -80°C .

For structural studies, nearly full-length (residues 25–304) and Δ TPR (166–304) constructs of mouse CHIP were cloned by PCR from mouse cDNA, inserted with an appended N-terminal His₆-tag and poliovirus 3C protease recognition sequence into pRSETA, expressed in *E. coli* BL21(DE3), and purified by metal affinity chromatography on Talon resin and by ion exchange on S-Sepharose. Fractions were treated with PreScission protease (20 U/mg protein, Pharmacia) to remove the N-terminal His₆ tag, reappplied to Talon resin, and then applied to a Superdex 200 PG gel-filtration column equilibrated in 20 mM Tris (pH 7.5), 300 mM NaCl, 1 mM EDTA, and 5 mM DTT. GST-Ubc13 and GST-Uev1a fusion proteins were purified by ion exchange on Q-Sepharose and immobilized on GST beads. Ubc13 or Uev1a was then released by PreScission cleavage and further purified on a Superdex 75 PG gel-filtration column.

Cell Culture, Transfection, and Cell Lysis

293 cells were cultured in 15 cm dishes in Dulbecco's modified Eagle's medium with 10% (v/v) fetal calf serum (FCS) and extracted with lysis buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.1% [v/v] 2-mercaptoethanol, 1% [w/v] Triton X-100, and complete proteinase inhibitor cocktail [Roche]). After centrifugation for 15 min at 18,000 \times g, the supernatant (termed cell extract) was decanted. Cells were transfected with polyethylenimine as described (Durocher et al., 2002).

Identification of Proteins in 293 Cell Extracts

Cell extract (40 ml) was incubated for 3 hr at 4°C with 0.5 ml packed glutathione Sepharose beads (Amersham) and GST-Ubc13 (23 nmol) and Uev1a (23 nmol). As a control, glutathione Sepharose was added to GST (23 nmol) and the same amount of 293 cell lysate. Beads were washed with 50 ml lysis buffer containing 250 mM NaCl and 25 ml of 50 mM Tris-HCl (pH 8), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 1 mM benzamidine, and 0.2% (w/v) Triton

(D) As in (A) but with Ubc13 added to an N-terminally truncated CHIP construct. The affinity remains micromolar, but the stoichiometry ($N = 0.8$) shows that two Ubc13 molecules are able to bind to each CHIP dimer in the absence of the TPR domains.

(E) N-terminal His-tagged Hsp90 was incubated with CHIP and/or Ubc13, as indicated. Hsp90 was precipitated by addition of TALON resin in 300 μ l of buffer containing 50 mM Tris (pH 7.5), 300 mM NaCl, 0.5 mM TECP, 5 mM Imidazole, 0.1% NP40, and 1 mg/ml BSA. Washed beads with bound proteins were boiled in SDS buffer, separated by SDS-PAGE, and visualized by immunoblotting with antibodies to Hsp90 (top), CHIP (middle), and Ubc13 (bottom). Ubc13 is clearly coprecipitated only when CHIP is present, confirming formation of a CHIP-bridged complex as shown in (F).

(F) Schematic of CHIP-mediated ubiquitylation of an Hsp90 bound client protein. The client protein (magenta) bound to the Hsp90 dimer is polyubiquitylated (red) by a ubiquitin-conjugating enzyme (yellow) bound to one of the U box domains of CHIP (blue). CHIP functions as a "Y" adaptor, coupling a single ubiquitylation system to a dimeric chaperone.

X-100, resuspended, incubated for 16 hr at 4°C with 4 µg PreScission protease, and centrifuged. Supernatants were concentrated and dialyzed against 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1 mM EGTA, 0.1% (v/v) mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF, and 50% (v/v) glycerol. Mass spectrometry was carried out by using an Applied Biosystems (Foster City, CA, USA) 4700 Proteomics analyzer, and the data searched by using the Mascot program (Perkins et al., 1999).

Crystallization, Data Collection, and Structure Determination

For the heterotrimeric complex, mCHIP(166–304), Ubc13, and Uev1a were combined in a 1:1:1 molar ratio, incubated for 30 min, and concentrated to ~10 mg/ml by ultrafiltration. Crystals were grown by vapor diffusion at 20°C against 20% (w/v) PEG2000 MME, 100 mM Tris-HCl (pH 7.0). For the full CHIP structure, mCHIP was mixed with human Hsp90α C-terminal peptide (NH₃⁺-DDTSRMEEDV-COO⁻) at a 1:3 molar ratio, respectively, incubated for 30 min at 4°C, and concentrated to ~10 mg/ml by ultrafiltration with a 5K Vivaspin concentrator. Initial multiple crystals were grown by vapor diffusion at 20°C against 30% w/v PEG4000, 100 mM Tris (pH 7.5), and 200 mM lithium sulfate. Subsequent streak seeding into solutions of 16% w/v PEG4000, 100 mM Tris (pH 7.5), and 400 mM lithium sulfate produced single plates. Crystals were harvested into reservoir solution with addition of glycerol (20% v/v) before flash cooling to 100K. X-ray data were collected on beamline ID14-eh3 at the ESRF, Grenoble, from single crystals, and processed by using MOSFLM (Leslie, 1995) and the CCP4 package (CCP4, 1994). Both types of crystals had space group C2, with four copies of the complex in the asymmetric unit for the heterotrimeric CUU complex and two copies of the dimer in the asymmetric unit of the full CHIP complex. The structure of the heterotrimeric complex was solved by molecular replacement with Phaser (CCP4, 1994) using human Ubc13-Mms2 (PDB code: 1JAT) as search model, and the resulting CHIP U box structure was then used along with the TPR domain from PP5 (PDB code: 1A17) as search models to solve the full CHIP-peptide complex structure. Both structures were built into difference Fourier density by using O (Jones et al., 1991) and COOT (Emsley and Cowtan, 2004) and refined with CNS (Brunger et al., 1998) and REFMAC (CCP4, 1994). Crystallographic statistics are given in Table 1.

Isothermal Titration Calorimetry

Heats were measured on a MSC system (Microcal), with proteins in 40 mM Tris (pH 8.0), 1 mM EDTA, and 50 mM NaCl at 30°C. For CHIP interactions, 10 aliquots of 27 µl of 200 µM CHIP were injected into 15 µM Hsp90 β or 300 µM PEP5 (NH₃⁺-MEEVD-COO⁻), PEP7 (NH₃⁺-SRMEEDV-COO⁻), or PEP10 (NH₃⁺-DDTSRMEEDV-COO⁻) were injected into 30 µM CHIP. For Ubc13 interactions, 10 aliquots of 27 µl of 300 µM Ubc13 were injected into 30 µM CHIP or CHIP[166–304] or 27 µl aliquots of 300 µM CHIP were injected into 30 µM Ubc13. Heats of dilution were determined in a separate experiment and the corrected data fitted with three floating variables: stoichiometry, binding constant, and change in enthalpy of interaction. For the Hsp90-PEP5 experiment, the stoichiometry was fixed at 1.0.

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Accession Numbers

Coordinates and structure factors have been deposited in the Protein Databank under 2C2L and 2C2V.